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CONTRACTING ORGANIZATION: Lawrence Livermore National Laboratory
Livermore, California 94550

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13. ABSTRACT ***(Maximum 200 Words)***

Understanding DNA repair mechanisms for ionizing radiation (IR)-induced DNA damage and having prior knowledge of a patient's IR-specific repair capacity will help determine which patients will be most responsive to radiation therapy and design more effective treatment regimes. The objective of this work has been to define the contributions of the mammalian protein Apel, and other candidate nucleases, to the repair of IR-induced genetic damage. We are currently constructing cell lines that lack Apel protein and will determine the sensitivity of these mutant cells to various DNA-damaging agents, particularly IR. We have constructed a hamster CHO cell line that has conditional expression of the human Apel protein under the control of a tetracycline-responsive promoter (Tet-off system of Clonetech). These cells are being used in gene targeting DNA transfection experiments to obtain a knockout mutation in *APE1*. Extensive analysis has been done to design highly sensitive PCR (polymerase chain reaction) screening procedures to identify and recover cells have *APE1* recombination events. So far, we have seen numerous gene targeting events that involve recombination of the right arm, but not the left arm, of the targeting vector. Ongoing studies are focused aggressively on getting the knockout mutant and performing extensive characterization of its radiation responses.

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Project Title: Repair Machinery for Radiation-Induced DNA Damage

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INTRODUCTION

Current methods to eradicate breast cancer and preserve normal tissue involve total mastectomy followed by radiotherapy. However, the use of postoperative radiotherapy has produced mixed results. While some randomized studies indicate that there is no major benefit from postmastectomy radiation treatment, there exists a subgroup of patients that benefit from this type of strategy. Thus, it is imperative that we design effective prognostic tools to identify patients that will profit, as well as those that may be harmed, by adjuvant radiotherapy. Ionizing radiation (IR) is cytotoxic to cells largely because it introduces lethal genetic damage. Thus, understanding the repair mechanisms for IR-induced DNA alterations and having prior knowledge of a patient's radiation-specific repair capacity will help design more effective treatment regimes and help determine which patients will be most responsive to radiation exposure. We intend to identify and characterize proteins involved in correcting the most abundant radiation-induced DNA damages. Such studies will provide the necessary tools (both specific DNA repair genes and biochemical assays) for predicting an individual's repair capacity and thus potential IR sensitivity (genetic predisposition).

BODY

Objective 1: To construct an *APE1* gene knockout cell line and examine the role of Ape1 in DNA repair

Background. Radiation induces an array of DNA damages, including abasic lesions and strand breaks harboring 3′-blocking termini such as phosphoglycolate and phosphate groups (1-3). The human Ape1 protein has been shown to incise at AP sites and remove a subset of 3′-damages, as well as

to stimulate the DNA-binding activity of several oncoproteins (e.g. p53, Fos and Jun) in vitro (4-7). Yet despite the basic understanding of the biochemical properties of Ape1, the in vivo function of the protein remains largely unclear, particularly as it relates to IR protection. The production of *APE1* knockout cell lines represents an essential step towards defining the biological contribution of this mammalian protein.

Strategy for isolating a knockout mutation of APE1. We predict that Ape1-deficient cells will demonstrate a quantitatively significant defect in the repair of certain DNA damages, but of which damages needs to be determined. We have selected Chinese hamster CHO cell line as the system for producing knockout mutations, since they present the many advantages described in the original proposal. As reported previously, we determined that *APE1* is a single copy gene in AA8 CHO cells and constructed the targeting vectors LARA.TK and LARA1 (Figure 1, see Appendix). These vectors are designed to delete a portion of the Ape1 C-terminal domain known to be essential for nuclease activity. We now discuss our progress toward isolating a knockout cell line (see Table 1 in Appendix for summary of experiments).

In successful gene targeting experiments being conducted by the new PI (L. Thompson) in other projects, it became clear that the targeting efficiencies were much lower than had been expected based on the one published report of a knockout mutation in the *ERCC1* gene (8). In that study the *ERCC1* knockout frequency was ~3×10^{-3} of the colonies surviving transfection. We obtained gene knockouts in the *XPD*, *FANCG*, and *RAD51C* genes at frequencies of 10^{-3} to 10^{-4}. To recover knockout events at these low frequencies, a screening strategy was devised that utilizes very sensitive PCR (polymerase chain reaction) procedures to identify a positive pool of colonies that contains a single mutant knockout colony among several hundred colonies. Once a positive pool of colonies/clones is identified, the mutant cells are recovered as pure clones in a two-step procedure that involves first screening small subpools and then screening individual clones from a positive subpool. This procedure has worked for the *XPD*, *FANCG*, and *RAD51C* genes.

Improvements in the screening procedure. Earlier targeting experiments for the *APE1* gene were hampered by the fact that genomic nucleotide sequence information was available for the left arm of the targeting vector but not the right arm (Fig. 1). Therefore, experiments were done to determine the nucleotide sequence of the flanking chromosomal region for the right arm. Primers for PCR reactions that would detect right-arm events were then made and verified. With PCR primers established that should detect both left-arm and right-arm recombination events for the targeting vector, the series of

experiments shown in Table 1 was performed. Although we have not yet succeeded in identifying a knockout mutation, we have observed partial recombination events between the targeting vector and the *APE1* gene. These partial events, referred to as type 3 events (9), occur when one arm of the vector is extended by an homology-dependent replication interaction with the *APE1* gene, followed by random integration of this sequence-extended vector. In addition to our standard DNA transfection procedures using electroporation to introduce DNA into cells, we also made modifications that we thought might improve targeting efficiency. The first was treatment of the linearized targeting vector with a 5′ to 3′ exonuclease digestion to generate single-stranded tails, which might mimic recombination intermediates (10). The second modification was the initial trasfection of the cells with a mammalian expression vector containing the dominant-negative form of human mismatch repair protein, hMSH3, in attempt to inhibit the cellular system of preventing recombination between sequences with imperfect homology. It was found that pre-treatment of the linearized targeting vector had a negative effect, reducing transfection efficiency, so it's employment was discontinued.

In experiment 4, we detected for the first time right-arm events but no recombination on the left arm. One possible explanation for the failure to see knockout events (e.g. Expts. 1-3) is that there are one or more mutations in the targeting vector, resulting in imperfect homology between the arms and the chromosomal DNA. We reasoned that disrupting the base-base mismatch recognition component of the mismatch repair system might allow the recombination reactions to proceed in the presence of a mutation. Therefore, the human mismatch repair protein hMSH3, whose overexpression has been shown to drastically reduce the mispair recognition and repair of the mismatch repair system in human cells (11), was introduced into the cells 24 or 48 h before introducing the targeting vector. In experiments 5-7, we detected seven more right-arm events but no knockout events. However, these right-arm events are encouraging because they mean that we have a specific interaction between the vector and the gene of interest. In experiments with other genes in other projects, the percentage of true knockout events is about 10-30% of the recombination events that occur on only one arm of the vector. Thus, we could have expected to identify a knockout event by now, having screened >30,000 transfection colonies. There remains the possibility that knockout events are occurring but we are not detecting them in the PCR screen on the left arm. This is a difficult issue to address in a foolproof manner because the positive control reactions with the PCR primers only imperfectly mimic the actual knockout event. An alternative possibility is that for unknown reasons *APE1* knockout recombination is very

inefficient compared with other genes, at least for the particular targeting vector configurations we are currently using.

Next innovations to improve targeting for knockout events. The next modification we will test, as a means to improve the ratio of targeted to nontargeted transfection events, involves the use of an inhibitor of the enzyme poly(ADP-ribose) polymerase (PARP). PARP is involved in DNA break recognition, particularly single-strand breaks (12,13). A study using the PARP inhibitor 3-methoxybenzamide found substantial improvement in targeting efficiency although there was some reduction in the yield of targeting events (14). This study performed targeting with the *APRT* gene using a simple drug selection to quantify the events. We had been familiar with the *APRT* gene and this work, but have not tried it because there is not a very clear rationale as to why it should work. To test whether these improved conditions for targeting the *APRT* gene will apply to *APE1* targeting, we will need to transfect larger numbers of cells than in past experiments. However, the number of colonies screened will probably be reduced because of higher enrichement (much lower frequency of random integration).

Objective 2: To characterize hYjeR and hXPMC2/Hem45 as 3' to 5' exonucleases and 3'-repair enzymes

This aim was completed last year as described in last year's progress report, according my recent telephone conversation with Dr. Wilson, the former PI.

KEY RESEARCH ACCOMPLISHMENTS

- Manuscript accepted for publication on the characterization of APE1-overexpressing cells
- Identified the first gene targeting events at the APE1 locus as a major step toward getting a full gene knockout; optimized PCR screening to detect recombination events for both arms of the targeting vector

REPORTABLE OUTCOMES

- Schild, L.J., K.W. Brookman, L.H. Thompson and D.M. Wilson III. (2002) Ape1 as a Rate-Limiting Factor in Cellular Resistance to DNA-damaging and Anti-cancer Agents. *Somat. Cell Mol. Genet.* In press.

CONCLUSIONS

Understanding the repair mechanisms for IR-induced DNA damage and having prior knowledge of a patient's radiation-specific repair capacity will help

determine which patients will be most responsive to radiation therapy and design more effective treatment regimes. The objective of this work has been to define the contributions of the mammalian protein Ape1, and other candidate nucleases, to the repair of IR-induced genetic damage. We are currently constructing cell lines that lack Ape1 protein and will determine the sensitivity of these mutant cells to various DNA-damaging agents, including IR. Future studies will focus on obtaining accurate quantitative determinations of the overall contribution of Ape1 to 3′-damage repair and IR protection and less on the search for alternative 3′-repair mechanisms, which were discussed in last year's report.

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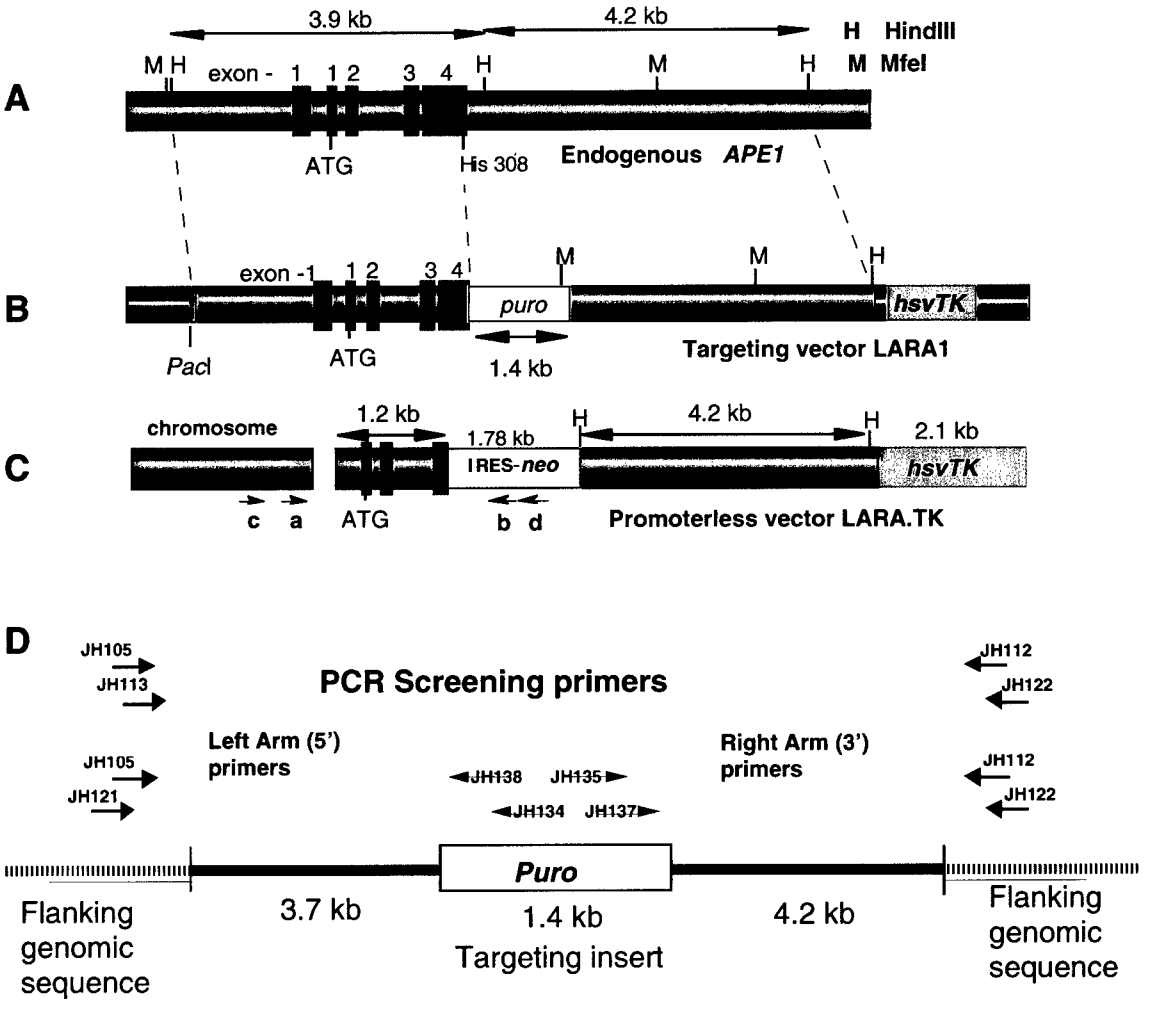
APPENDICES

Table 1. Summary of APE1 Gene Targeting Experiments from 2/22/02 to 6/31/02
(The vector pLARA1 was used in all experiments except Expt. 8)

Expt	Date	Colonies/plate	Dishes	Total colonies	Right-arm events	Left-arm events
1	2/22/02	55.2	12	662	0	0
2	3/6/02					
	E1:	314	4	1256	0	0
	E2:	265	5	1325	0	0
	E3:	238	3	714	0	0
	E4:	246	8	1968	0	0
	subtotal:		24	5,263	0	0
3	3/25/02 (only E1 Exo-treated)					
	E1:	99	3	297	0	0
	E2:	125	10	1250	0	0
	E3:	73	30	2376	0	0
	subtotal:		43	3,923	0	0
4	3/28/02 (only E1 Exo-treated)					
	E1:	80	20	1600	0	0
	E2:	316	22	6960	2	0
	subtotal:		42	8,560	**2**	0
5	4/30/02 (hMSH3 via electroporation; 24 h)					
	E1:	153	11	1683	2	0
	E2:	153	11	1683	3	0
	subtotal:		22	3,366	**5**	0
6	5/9/02 (hMSH3 via electroporation; 48 h)					
	E1:	181	11	1991	0	0
	E2:	185	9	1665	0	0
	E3:	189	10	1890	2	0
	E4:	168	10	1680	0	0
	subtotal:		40	7,226	**2**	0
7	6/13/02 (hMSH3 via Lipofectamine, 24 h)					
	E1:	230	8	1820	≥ 2	0
	E2:	230	8	1820	≥ 2	0
	subtotal:		16	3640	**4**	0
8	6/13/02 pLARA.TK (G418)					
	E1:	124	8	992	0	0
	E2:	124	8	992	0	0
	total:		16	1984	0	0
	TOTAL Overall			**34,624**		

Figure 1. Schematic of *APE1* locus, gene knockout vectors, and primer positions for PCR analysis. (**A**) The 1.8- kb coding region of *APE1* gene encodes a 318 amino acid protein. The exons are indicated in red boxes and a critical residue for nuclease activity (His 309) is indicated. *Hin*dIII and *Mfe*I recognition sites are marked by H and M, respectively. (**B**) The LARA1 targeting vector contains an interruption in exon 4 with the puromycin (*puro*) gene, thereby eliminating His 309. The 4.2-kb right arm is positioned between *puro* and the negative selection marker, *hsvTK,* which helps suppress random integration. (**C**) A promoterless targeting vector, LARA.TK, utilizes the endogenous *APE1* promoter to drive expression of the neomycin (*neo*) gene, which interrupts *APE1* in exon 3. The 1.2-kb left arm terminates *APE1* translation at residue 87, and eliminates Cys 93, a key residue for the redox activity of Ape1 protein. (**D**) Successful amplification has been performed with the right arm screening primers (for the type three events we've found) and the trans-locus primers. The left Arm primer pairs diagramed are those presumed to be the best choices of the many tested, based on low background and amplification success with control primers/plasmids.



PERSONNEL

Role on Project	Name	Degree	Scientific Discipline	Institutional Affiliation
PI	Lawrence H. Thompson	Ph.D.	Radiation Biology & Molecular Genetics	Lawrence Livermore National Laboratory, Univ. of California (LLNL-UC)
Post-doctoral Scientist	John Hinz	Ph.D.	Molecular & Cellular Biology	LLNL-UC
Consultant	David Wilson (previous PI)	Ph.D.	Protein Biochemisty	National Institute on Aging (Baltimore, MD)

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

Lawrence Hadley Thompson	**DNA Repair Group Leader**/Sr. Biomedical Scientist

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
University of Texas, Austin, Texas	B.S.	1963	Physics (Magna cum laude)
Univ. of Texas Graduate School Biomedical Sci., Houston	M.S.	1967	Biophysics (Radiat. Biol.)
Univ. of Texas Graduate School Biomedical Sci., Houston	Ph.D.	1969	Biophysics (Radiat. Biol.)
Ontario Cancer Institute, Toronto, Ontario, Canada	Postdoctoral	1969-71	Somatic cell genetics

A. Positions and Honors

PROFESSIONAL POSITIONS

1964-69	Predoctoral NIH-supported Fellow in M.S. and Ph.D. Programs, University of Texas Graduate School of Biomedical Sciences, Houston, TX
1969-71	Post-doctoral Fellow, Ontario Cancer Institute, Toronto, Ontario, Canada.
1971-73	Staff Physicist, Ontario Cancer Institute, Toronto, Ontario, Canada.
1973-Present	Senior Biomedical Scientist, Biology and Biotechnology Research Program, Lawrence Livermore National Laboratory, University of California, Livermore, CA
1990-Present	Adjunct Professor, Department of Radiation Oncology, School of Medicine, University of California, San Francisco, CA
1991-Present	Group/Team Leader, DNA Repair Team, Biology and Biotechnology Research Program, Lawrence Livermore National Laboratory, Univ. of California, Livermore, CA

HONORS

Phi Beta Kappa (Arts and Sciences), 1963
Phi Eta Sigma (Physics), 1963
Sigma Pi Sigma (Freshman men), 1960
Predoctoral NIH fellowship, Dept. of Radiation Therapy, M.D. Anderson Hospital., Houston, TX, 1964-1969

Journal Editorial Boards

1986 - Present	Molecular and Cellular Biology
1978 - Present	Somatic Cell & Molecular Genetics
1982 - Present	Mutation Research
1985 - through 1996	Mutagenesis
1993 - through 1998	Environmental & Molecular Mutagenesis

Distinguished Alumnus Award, May 1996; The University of Texas Graduate School of Biomedical Sciences, Houston, TX (first such award given by the school in its 34-yr history)

Member, Site Visit for National Cancer Institute Laboratory of Molecular Carcinogenesis, Nov. 19-91, 1997, Bethesda, MD

Member, Review panel of DOE's Low-Dose RFA in June 2-3, 1999, Arlington, VA

Member (ad hoc) of NIH Radiation Study Section, Feb 28-Mar 1, 2000

Member of NIH/NCI Program Virtual Project Site Visit Panel, Mass. Gen. Hospital, Boston, MA, Oct 3-5, 2001

Examples of invited presentations:

Speaker at meeting on Translational Opportunities in/from DNA Repair Research, sponsored by National Cancer Institute, May 5-7, 1999, Chicago, Ilinois

Speaker at Analysis and Prevention of Carcinogenesis in Animal Models for DNA Repair Related Genes, Tokyo, Japan, Sept. 21-23, 1999

Speaker at Gordon Conference on DNA Repair, Ventura, CA Jan., 2001

Speaker at Gordon Conference on Radiation Oncology, Ventura, CA, Feb. 2001

Ann. Mtg. Am. Chem. Society, San Diego, CA, Apr 2, 2001

Workshop on "The Role of DNA Damage Response Defects in Neurodegenerative Diseases", Tarrytown, NY, July 29-Aug 1, 2001

Societies

American Association for the Advancement of Science
Environmental Mutagen Society, 1982-present; Council Member 1985-1988
Genetic and Environmental Toxicology Association of Northern California, 1985-present
Radiation Research Society, 1995-present

B. PUBLICATIONS (total ~165; selected from last 5 years, includes invited reviews in books and journals)

Thompson, L. H. (1998) Nucleotide excision repair: its relation to human disease. *In* DNA Damage and Repair, Vol. 2: DNA Repair in Higher Eukaryotes, J.A. Nickoloff and M. Hoekstra (Eds.), Humana Press, Totowa, NJ, Chapter 18, pp. 335-393.

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Thompson, L.H. and M.G. West. (2000) XRCC1 keeps DNA from getting stranded. Mutat. Res. 459: 1-18 (review).

Takata, M., M. S. Sasakai, E. Sonoda, T. Fukushima, C. Morrison, J. Albala, S. Swagemakers, R. Kanaar, L. H. Thompson, and S. Takeda. (2000) The Rad51 paralog Rad51B promotes homologous recombinational repair. Mol. Cell. Biol. 20: 6476-6482.

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Kadkhodayan, S., F. Coin, E.P. Salazar, J.W. George, J.M. Egly, and L.H. Thompson (2001) Codominance associated with overexpression of certain XPD mutations, Mutat. Res. 485: 153-168.

Takata, M., M. S. Sasaki, S. Tachiiri, T. Fukushima, E. Sonoda, D. Schild, L. H. Thompson, and S. Takeda. (2001) Chromosome instability and defective recombinational repair in knockout mutants of the five Rad51 paralogs. Mol. Cell. Biol. 21: 2858-2866.

Tebbs, R. S., E. P. Salazar, and L. H. Thompson. 2001. Identification of IRC170-induced *XPD* mutations in UV-sensitive CHO cells. Environ. Mol. Mutagen. 38: 111-117.

George, J., E. Salazar, M. Vreeswijk, J. Lamerdin, J. Reardon, M. Zdzienicka, A. Sancar, S. Kodkhodayan, R. Tebbs, L. Mullenders, and L. Thompson. 2001. Restoration of nucleotide-excision repair in a helicase-deficient *XPD* mutant from intragenic suppression by trichothiodystrophy mutation(s). Mol. Cell. Biol. 21: 7355-7365.

Fujimori, A., S. Tachiiri, E. Sonoda, P. K. Dhar, M. Hiraoka, S. Takeda, L. H. Thompson, and M. Takata. 2001. Rad52 substitutes for the Rad51 paralogs in maintaining chromosomal integrity in vertebrate cells. EMBO J. 20: 5513-5520.

N. Liu, D. Schild, M.P. Thelen, L.H. Thompson, Involvement of Rad51C in two distinct protein complexes of Rad51 paralogs in human cells, Nucleic Acids Res. 30 (2002) 1009-1015.

C. Wiese, D.W. Collins, J.S. Albala, L.H. Thompson, A. Kronenberg, D. Schild, Interactions involving the Rad51 paralogs Rad51C and XRCC3 in human cells, Nucleic Acids Res. 30 (2002) 1001-1008.

M. Takata, S. Tachiiri, A. Fujimori, L.H. Thompson, Y. Miki, M. Hiraoka, S. Takeda, M. Yamazoe, Conserved domains in the chicken homologue of BRCA2, Oncogene 21 (2002) 1130-1134.

C. Research Support. List selected ongoing or completed (during the last three years) research projects (federal and non-federal support). Begin with the projects that are most relevant to the research proposed in this application. Briefly indicate the overall goals of the projects and responsibilities of principal investigator identified above.

ONGOING

(L. Thompson, PI) 10/1/01-9/30/04 60% effort
DOE/OBER KP1102020, SCW0008 $470,000 direct/yr
Assessing Biological Function of DNA-Damage Response Genes
The main goal of this project is to construct knockout mutants in CHO cells for a variety of DNA damage-response genes, with emphasis on genes in homologous recombination and base excision repair.
OVERLAP: None.

(L. Thompson, PI)
R01 CA52679-10 4/1/98-4/30/03 10%
NIH/NCI $231,200 direct/yr
Genetic Analysis of Nucleotide Excision Repair
The major goal of this project is to characterize the role of the ERCC2/XPD helicase in nucleotide excision repair.
OVERLAP: None

(L. Thompson, PI)
R01 CA89405-01 1/24/01-12/31/04 20%
NIH/NCI $180,000 direct/yr
The Fanconi Anemia Gene Pathway in Radiation Responses
The goal is to characterize the role of the FANCG protein in response to DNA damage.
OVERLAP: None

(John Tainer, PI)
L. Thompson, Project 4 Leader
1 P01 CA92584-01 9/27/01-8/31/06 10% effort
NIH/NCI $136,000 direct/yr
Structural Cell Biology of DNA Repair Machines, Project 4: Multi-Component Complexes in Homologous Recombination. The goal is to determine molecular structures of Rad51 paralogs.
OVERLAP: None.